THE ISOLATION OF SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES FROM BOVINE CEREBRAL CORTEX

Joseph Anthony DiVerdi

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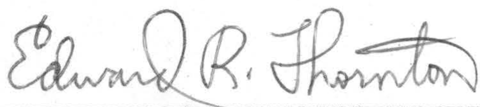

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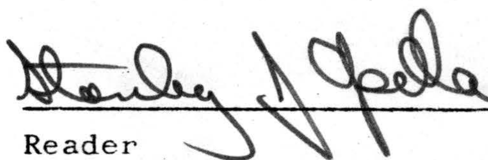

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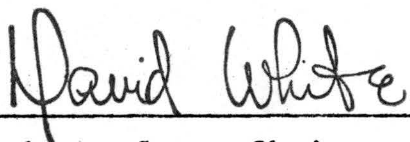

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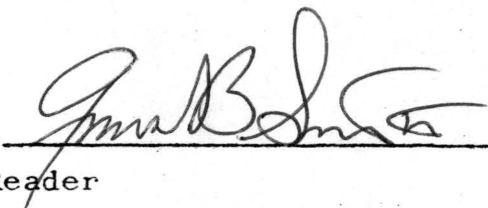

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ABSTRACT

A method is described for the preparation of synaptosomes and synaptic plasma membranes from Bovine cerebral cortex. After homogenization of the tissue in isotonic sucrose, differential centrifugation yielded a crude mitochondrial fraction (P_2) which was purified by centrifugation on a Ficoll-sucrose gradient. Assay of occluded Lactate Dehydrogenase and electron microscopy confirmed that synaptosomes survived the fractionation procedure and appeared in the P_2B and P_2C fractions, sedimenting to a density of approximately 1.117. The final membrane preparation had a specific 5'-nucleotidase activity (units/mg protein) over 10 times higher than the brain homogenate. Membrane fragments derived from outer mitochondrial membranes and microsomes appear to be the major contaminants. Gel electrophoresis revealed proteins migrating at approximately the same rate as contractile proteins.

LIST OF ABBREVIATIONS

ACHE- acetylcholinesterase
Acid P.- acid phosphotase
Alk. P.- alkaline phosphotase
AMP- adenosine monophosphate
ATP- adenosine triphosphate
BIS- methylene-bis-acrylamide
BME- β-mercaptoethanol
CMF- crude mitochondrial fraction
EM- electron microscopy(scanning-SEM; transmission-TEM)
ER- endoplasmic reticulum
INT- ρ-iodinitrotetrazolium violet
LDH- lactate dehydrogenase
MAO- monoamine oxidase
MP- osmotic shock medium (1mM $MgCl_2$, 1mM K_2HPO_4, pH 7.6)
NAD- nicotine adenine dinucleotide
5'-nuc- 5'-nucleotidase
PAGE- polyacrylamide gel electrophoresis
PMS- phenathiozine methosulfate
PMSF- phenylmethylsulfonylfluoride
SDH- succinate dehydrogenase
SDS- sodium dodecyl sulfate
SMP- homogenization medium (0.32 M sucrose in MP)
SPM- synaptic plasma membranes
TCA- trichloroacetic acid

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INTRODUCTION

Since the pioneering work of Whittaker (1959), Whittaker *et al*. (1964), and De Robertis *et al*. (1961), many authors have described preparations of pinched-off nerve endings commonly called "synaptosomes". Most investigations use porcine or rat brain which is homogenized in isotonic saline (0.9%) or sucrose (0.32 M). This step takes advantage of the fortuitous property of the club-like presynaptic nerve endings to respond to the homogenization by tearing or snapping off the attached nerve cell and resealing to form closed vesicles containing the main structural and functional features of the synapse. The utility of these particles is not only the ease with which they can be manipulated (as one would work with cells in culture or bacteria) but their capacity to perform like the whole cells from which they are derived with high linear respiration producing lactate and amino acids, generating ATP and phosphocreatine, and accumulating K^+ and extruding Na^+ against a concentration gradient (Ling, Abdel and Latif, 1968; Bradford, 1968; Bradford and Thomas, 1969).

Synaptosomes and synaptic plasma membranes are useful for studying neurotransmission since the SPM is the actual

locus of contact between neurons. They can also be used to study permeability in membranes since the depolarization phenomenon is governed by Na^+ and K^+ pumps.

The first step in the isolation of synaptosomes is rate or differential centrifugation which will separate the organelles roughly on the basis of sedimentation coefficient (see Fig. 1).

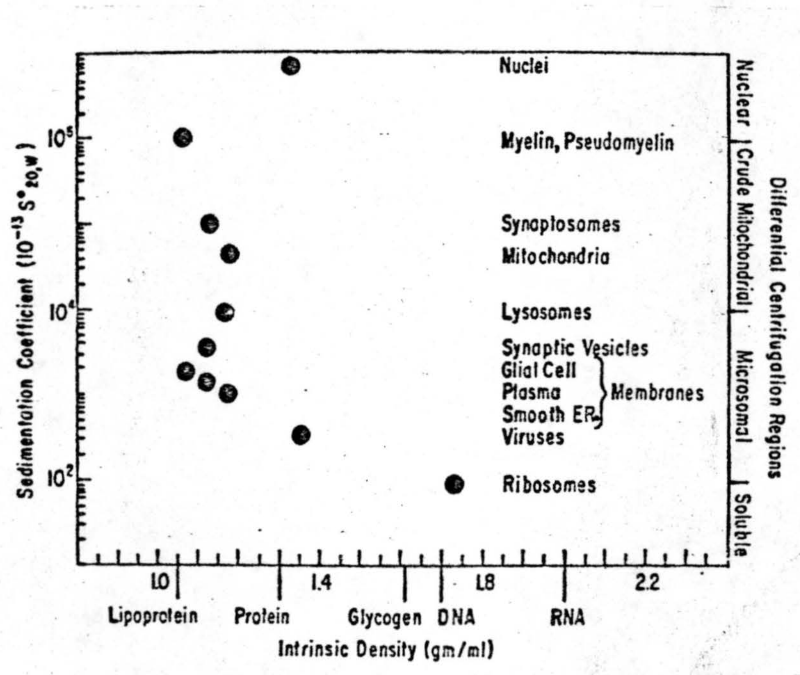


Fig. 1. Size and density of subcellular particles in a typical brain homogenate, from Appel *et al.* (1972).

Overall, rate centrifugation has relatively low resolution (it can, however, be improved by washing the fractions and recycling the washes. Nevertheless, it has proven extremely

useful principally because it can be applied to the separation of particles whose sedimentation coefficients differ by orders of magnitude (Anderson, 1966). The "crude mitochondrial fraction", named so because the centrifugation conditions corresponded to the mitochondrial fraction obtained from liver, obtained is an extremely heterogeneous population consisting of mylen fragments, various membrane fragments, synaptosomes and mitochondria. However, it can be further resolved by isopycnic density gradient centrifugation. This separation takes advantage of the intrinsic densities of the various components of the CMF but is not always used in an equilibrium separation. Some investigations (Morgan *et al.*, 1971) achieved a better separation by allowing the synaptosomes to reach equilibrium and stopping before the other components have done so. The major variations of this step are the choice of gradient material and the (dis)continuity of the gradient. Previously (Whittaker *et al.*, 1964; Whittaker, 1959; De Robertis *et al.*, 1961; Levitan *et al.*, 1972), a discontinuous sucrose gradient was utilized but this approach has been largely abandoned due to the shrinking of synaptosomes in hypertonic sucrose resulting in poor morphology, banding

at densities closer to mitochondria, and difficulty in osmotic shocking of the recovered synaptosomes. An alternative is to substitute th high molecular weight polysaccharide Ficoll for sucrose. The usual procedure is to use varing concentrations of ficoll in 0.32 M sucrose to achieve different densities under iso-osmotic conditions (Morgan et al. 1972; Cotman and Matthews, 1971).

Garey et al. (1972) have made several interesting observations on this subject. First, they maintain that commercially available Ficoll contains numerous low molecular weight components and must be purified before use in order to take full advantage of the iso-osmolar condition. Secondly they note that sucrose gradients which have the undesirable feature of producing osmotic damage, seem to yield better resolution of the particulates than the Ficoll gradient. They posit the reason for the superiority of the discontinuous sucrose gradient is that they in fact, linearize before use while Ficoll-sucrose gradients would require ten times the time given to the sucrose gradient to linearize to the same extent. Consequently, if continuous Ficoll-sucrose gradients were prepared in the proper profile, one could achieve iso-osmotic conditions with the same resolution as with the sucrose

gradients. It is unfortunate that they do not subject their preparation to rigorous enzymatic analysis but rely on electron microscopy (certainly not quantitative) for their conclusions.

Cotman and Taylor (1972) demonstrated an interesting approach to increase the resolution of the density gradient. They utilized the histochemical location of succinate dehydrogenase (SDH) in mitochondria to weigh down mitochondrial membranes with formazan produced by reduction of p-iodonitrotetrazolium violet (INT) by SDH and succinate. This results in lower contamination of synaptosomes by mitochondrial membranes.

At this point there is a divergence in procedure depending on the aim of the investigation. If the goal is to study metabolism or translocation of various substances then it is not uncommon to stop here and use the intact synaptosomes. The critisism to this is the above stated (im)purity of this fraction but little has been done to better the situation. In the discussion section of this thesis I will propose a method to further reduce non-synaptosomal contamination. The other direction would be to study the various components that make-up synaptosomes (i.e. synaptoplasm, synaptic vesicles

(SV), synaptic plasma membrane (SPM), synaptic junctional complexes (SJC), synaptic mitochondria). Advantage can be taken of the various sub-fractionation procedures which will necessarily also fractionate the non-synaptosomal contaminants but I must state the desirability of removing contaminants as early in the protocol as possible as shown by the improvements of the density gradient separation by scraping the brain tissues to remove visible myelin before homogenization (Garey *èt al.*, 1972 and this paper).

The next step in the preparation of synaptosomal organelles is osmotic shock of the synaptosomal fraction. As shown by Cotman and Matthews (1971) the condition of pH is crucial at this point. At neutral pH (pH 7.1) some 50% of cytochrome oxidase overlaps 89% of Na^{+}-K^{+} ATPase while at higher pH (pH 8.5) 95% of the mitochondria separate from 85% of the membrane. Ionic strength is also important. A highly positively charged form of Lactate dehydrogenase (LDH) is known (Fonnum, 1967) and is capable of binding to membrane under low ionic strength condition. This must not be taken too far because it is also shown by Whittaker *et al.* (1964) and by data presented here that high concentrations of divalent ions cause artifactual aggregation of paticles.

It is interesting to note the effect of Ca^{++} in shocking. Whittaker (1964) notes "the tendancy of [synaptic] vesicles to remain clumped together after release as though embedded in a sticky cytoplasm." Also DeRobertis _et_ _al_. (1963) stressed the presence of 10 umolar Ca^{++} (as $CaCl_2$) in the water used to rupture synaptosomes to influence clumping of subcellular particles and to effect retention of the [synaptic] vesicles, together with soluble proteins and enzymes, in the synaptosomes prior to their distribution. Their observation together with the finding of muscle-like contractile protein in synaptosomes is interesting in light of the proposed contractile protein aided model of neurotransmission (Berl _et_ _al_., 1973). It is possible that the "sticky cytoplasm" mentioned by Whittaker is in fact filaments of actin or tubulin (a micro-tubule protein), which are involved with the neurotransmission process.

Finally, for separating the disrupted particles a sucrose density gradient proves useful (Whittaker _et_ _al_., 1964; Morgan _et_ _al_., 1971; Levitan _et_ _al_., 1972; Cotman and Matthews, 1971). Ficoll is not necessary this time since the particles do not appear to be osmotically sensitive. As noted earlier, Cotman and Matthews (1971) achieve a good separation of SPM's

from mitochondrial membrane by the use of discontinuous sucrose gradients after conditions of alkaline osmotic shock. Complementing the use of discontinuous sucrose gradients, Cotman _et al_. (1968) utilize continuous sucrose (15 - 50%, w/w) zonal ultracentrifugation. They noted that when a CMF was shocked and placed on this gradient a particulate band was observed at 29 - 32% sucrose which was not present when an isotonically prepared CMF was applied to the gradient. EM analysis showed that this band contained free membrane with some synaptic thickenings. Na^+-K^+ ATPase was localized in this fraction with the concentration of mitochondrial and microsomal enzymes being low. They estimate the contamination at about 10 - 20%.

DeRobertis _et al_. (1973) utilize rate centrifugation in the separation of synaptosomal organelles after osmotic shock (as mentioned earlier, in the presence of $CaCl_2$). Their rationale is to minimize contact with sucrose. Their resolution is not as good as with others, more than likely because of the aforementioned limitation of rate centrifugation. [The subject of synaptosomes and SPM preparation has been recently reviewed by Morgan (1972) and Morgan and Gombos (1976).]

It is useful to use enzymes to assay contamination of

membrane preparations, since an enzyme with a specialized function is likely to have a specialized location. However, it is necessary to remain critical of the use of a marker enzyme since there are a number of complications which may arise regarding them: e.g., (1) during the disruption conditions markers may redistribute; (2) markers may be localized at more than one organelle; (3) markers may be activated or inactivated, partially or totally during fractionation; (4) a contaminant may be completely lacking in markers; (5) the true specific activity of a marker may not be known. Nevertheless, enzyme markers prove to be useful in assessing contamination and they can be used in conjunction with other methods (e.g., EM, PAGE) quite effectively. I will briefly discuss the markers used in neural membrane fractionation.

Lactate dehydrogenase has been long known as a cytoplasmic marker (Johnson and Whittaker, 1963), and in its occluded form is a good marker for intact synaptosomes (Marchbanks, 1967). 5'-nucleotidase is considered by Wallach and Winzler (1974) to be most specific for plasma membrane. Alkaline Phosphotase was found to closely follow Na^{+}-K^{+} ATPase which was most highly enriched in SPM fractions (Cotman and Matthews, 1971). However, it is not exclusively a plasma

membrane marker in brain because histochemical studies have found a high concentration of alkaline phosphotase activity in capillary walls (Friskman and Hayaski, 1962; Novikoff, 1967). By histochemical analysis ACHE is found associated with axonal and dendritic membranes and endoplasmic reticulum (Novikoff, 1967). The ER contains many enzymes but only a few have been used as markers. d-glucose-6-phosphotase and NADH-cytochrome c oxidoreductase are not exclusively located here (Wallach and Winzler, 1974). Acid phosphotase, β-glucaronidase, DNase, RNase, and aryl sulphatase are considered to be lysosome specific (Wallach and Winzler, 1974). Cytochrome oxidase and succinate dehydrogenase are believed to be markers for inner mitochondrial membrane (Sottocasa <u>et al.</u>, 1967). Monoamine oxidase is now accepted as a marker for the outer mitochondrial membrane after some considerable controversy (Smoley <u>et al.</u>, 1970).

This thesis describes a procedure whereby synaptosomes and synaptic plasma membrane are prepared using Bovine cerebral cortex. It is an attempt to optimize a preparation of these organelles for this new tissue. Synaptosomes have been prepared from many species and brain regions (see Jones, 1975, for review) but not this tissue and species. The

reasons for choosing bovine brain are: (1) its large quantities, i.e. 40 grams of grey matter from cortex can be routinely obtained from one 3/4 pound brain free of white matter; (2) economy; (3) availability, brain is routinely obtained at the slaughterhouse and placed on ice within twenty minutes of the time of death of the animal. The brain arrives at the laboratory within thirty minutes after that and is processed no longer than one hour after the death of the animal. (Preliminary experiments were performed and further experiments are outlined to attempt freezing of the brain to permit preservation of the brain tissue prior to fractionation.)

METHODS

Materials: The following chemicals were used as obtained: l-lactic acid, NAD, INT, PMS, succinic acid, eserine sulfate, antimycin-A, cytochrome-c, AMP type II, SDS 99.9+ %, sucrose grade I, bovine serum albumin 3x crystallized, ficoll type 400, poly l-lysine bromide type 1B, trizma base 99.9+ %, glycine, BME, bromphenol blue, Coomassie brilliant blue, PMSF from Sigma Co., St. Louis, Mo. S-acetylthiocholine, ρ-nitrophenylphosphate-disodium salt, ATP-disodium salt, methyl-ρ-aminophenolsulfate, acrylamide 99.9+ %, BIS 99.9+ %, glycerol from Aldrich Co., Metuchen, N.J. Inorganic salts were Baker Analyzed Reagent Grade, T.T. Baker Co., Phillipsburg, N.J. Water used was glass-distilled deionized on a mixed resin bed and stored in polyethylene. Propylene oxide, Epon 812, 300 mesh copper grids, osmium tetroxide, uranyl acetate were obtained from Electron Microscopy Scienes, Fort Washington, Pa. Tetraethylenemethylenediamide (TEMED) was obtained from Bio-Rad, Co., Richmond, Ca. Benzylamine was prepared as the hydrochloride by titrating the free amine (Sigma Co.) with HCL (conc) and recrystallizing the salt from absolute ethanol. Glutaraldehyde was purified

from a 25% aqueous stock solution (Aldrich, Co.) by repeated decolorization withcharcoal until the $OD_{235}/OD_{250}=$ 0.21.[1] The protein standards for electrophoresis were obtained as follows: phosphorylase A (Sigma Co.), chymotrypsinogen A (Miles), gluamic dehydrogenase (Miles), lysozyme (ICN), alcohol dehydrogenase (ICN).

1. personal communication, F. Rheingold, Electron microscopy labrotories, School of Medicine, University of Pa.

PREPARATION OF TISSUE FRACTIONS

Primary Fractions: All operations were conducted at 0-4 °C. Cerebral cortex was dissected by making cuts 1 cm apart, approximately 5 cm long in the brain and cutting back the surface layer 1/2 cm deep. This allowed removal of grey matter without contamination by white matter. The tissue was minced with scissors and homogenized in the homogenizing medium, SMP (0.32 M sucrose, 1mM $MgCl_2$, 1mM K_2HPO_4, adjusted to pH 7.6 with 1 N HCl) at a tissue concentration of 20% (w/v) in a Thomas type-C homogenizer (clearance 0.22 mm) operated at 900 rpm for 8 up and down strokes. The suspension was diluted with SMP to a tissue concentration of 10% (w/v) and samples were taken for assay. This homogenate was then subjected to the rate centrifugation scheme (Fig. 2) based on previous work (Morgan et al., 1971; Rodriguiez et al., 1967). This separation was routinely performed in a SS-34 or GSA rotor in a RC5 Sorvall superspeed refrigerated centrifuge. To separate P_3 and S_3 a type 42.1 rotor on a Spinco L-2 ultracentrifuge was used.

Ficoll-Succrose Density Gradient: (as in Fig. 3) The resuspended P_2 pellet was further fractionated into subfractions A,B,C and D by means of a discontinuous ficoll-sucrose

Fig. 2 Preparation of Primary Fractions

Homogenize bovine cerebral cortex in SMP, 20% (w/v) tissue conc., at 900 rpm, 8 strokes

↓

dilute to 10% (w/v) with SMP

↓

1000 G x 15min.

Pellet → washed once by resuspension and centrifuged at 1000 G x 15 min. → P_1

supernatant

washing → 1000 G x 15 min.

pellet ← (to P_1)

supernatant

↓

11,000 G x 25 min.

pellet → washed by resuspension and centrifuged at 11,000 G x 25 min.

washings → 100,000 G x 60 min.

pellet → resuspend in SMP (3 ml/ gm brain) → P_2

supernatant

pellet → P_3

supernatant → S_3

density gradient (Morgan _et al._, 1971; Cotman, 1972). The P_2 fraction was routinely layered on a gradient (38 ml/ tube) of equal volumes of 9,12 and 16% ficoll in 0.32 M sucrose (optionally with a 2 ml 2.2 M sucrose cushion). The gradients were spun in a Spinco L-2 ultracentrifuge using a SW 27 rotor at 55,000 G_{avg} (21,000rpm) x120 minutes. The interfaces were separated by carefully removing them with a Pasteur pipette. Each interface (and the pellet) was diluted with at least 5 volumes of SMP and pelleted at 40,000 G x 30 min., then resuspended in a small volume of SMP.

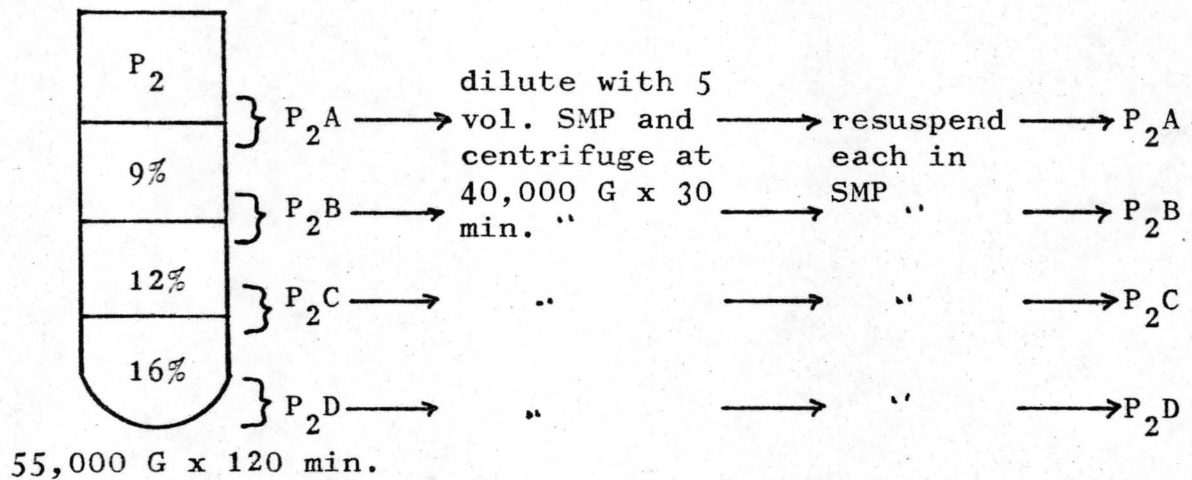


Fig. 3 Ficoll-Sucrose Density Gradient Centrifugation

Osmotic Shock of Fractions: (as in Fig. 4) The P_2B or P_2C pellets were disrupted by osmotic shock by mixing the fraction to be shocked with 9 volumes of MP (1mM $MgCl_2$, 1mM K_2HPO_4,

adjusted to pH 7.6 with 1N HCl), homogenized in a tight-fitting glass homogenizer, incubated 1 hour on ice and homogenized again. The suspension was then centrifuged at 40,000 G x 60 min. to yield a supernatant (W_s) and a pellet (W_p) which was resuspended in a small amount of MP.

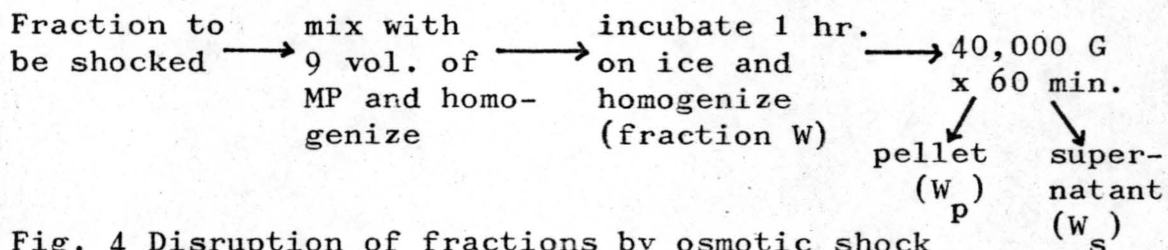


Fig. 4 Disruption of fractions by osmotic shock

Occasionally, it was desired to concentrate the W_s fraction. This could be accomplished by either of two methods: (1) make the solution 10% (w/v) in TCA (using a stock aqueous solution of 1 gm TCA per ml of solution), incubate for one hour at 0-4°C, pellet at 40,000 G x 60 min. and resuspend in a small volume of MP or (2) lyophilize the solution, resuspend in a small amount of H_2O and dialyze against MP.

Density Gradient Separation of Disrupted Fractions: W_p fractions were transferred to discontinuous sucrose density gradients consisting of equal volumes of 0.4,0.6,0.8, 1.0 and 1.2 M sucrose in MP. Gradients were immediately

centrifuged at 55,000 G_{avg} (21,000 rpm) x 2 hours in a SW 27 rotor. After centrifuging, fractions were separated as before, diluted with at least 5 volumes of MP and pelleted at 40,000 G x 60 min. and then resuspended in a small amount of MP.

Ficoll-Sucrose Density Gradients in the SZ-14 Rotor: In order to attempt a scale-up of this procedure the SZ-14 reorienting gradient zonal rotor (Sorval) was used. The rotor was loaded statically with 100 ml of P_2 suspension followed by 300 ml of each of 9, 12 and 16% ficoll in SMP and 300 ml of 2.2 M sucrose. The rotor was accelerated at a setting of 45 (arbitrary units) taking approximatiely 10 minutes to achieve 800 rpm and accelerated normally to 21,000 rpm (45,000 G_{avg}) for 3 hours. The rotor was allowed to decelerate to 800 rpm with braking then allowed to coast to a stop. The contents were pumped out and 20 ml fractions were collected and assayed for protein, ACHE and MAO.

Freezing Experiments: In one experiment, brains obtained at the meat packers were quickly frozen in liquid nitrogen to study the effect of quick freezing and storage of brains. P_1, P_2, P_3 and S_3 fractions were obtained and assayed for protein and ACHE.

ENZYME ASSAYS

Lactate dehydrogenase (EC 1.1.1.27) was assayed by following the reduction of NAD coupled with PMS and INT in the presence of l-lactate an 550nm (Bergmeyer and Brant, 1974). Succinate dehydrogenase (EC 1.3.99.1) was assayed by following the reduction of FAD coupled to PMS and INT in the presence of succinate at 550 nm (Nachlas, 1960). Acetylcholinesterase (EC 3.1.1.7) was assayed by measuring the activity inhibited by 10 ug ml^{-1} eserine sulfate with S-acetylthiocholine as a substrate at 412 nm (Ellman et al., 1961). Acid Phosphotase (EC 3.1.3.2) was assayed by following the hydrolysis of ρ-nitrophenol phosphate at 412 nm at pH 5.0 and Alkaline Phosphotase as in acid phosphotase at pH 9.5 (Cotman and Matthews, 1971). Monoamine Oxidase (EC 1.4.3.4) was assayed by following the oxidative deamination of benzylamine-HCl at 250 nm (Schnaitman et al., 1967). Na^{+}-K^{+} activated ATPase (EC 3.6.1.3) was assayed by measuring the ATPase activity activated by 24 mM KCl and 120 mM NaCl (Dahl, 1964). The reaction was terminated by the addition of the acid-molybdate reagent of the phosphate assay. Antimycin-insensitive NADH-cytochrome c oxidoreductase (EC 1.6.99.3) was assayed by following the reduction of cyto-

cytochrome c at 550 nm in the presence of 100 ug ml^{-1} of antimycin A (Dallner, 1966). 5'-nucleotidase (EC 3.1.3.5) was assayed by measuring phosphate release from adenosine 5'-phosphate (Cotman and Matthews, 1971). The reaction was terminated as in ATPase. Protein was removed prior to reading OD_{660} by centrifugation.

One unit of each enzyme was defined as the amount required to catalyze the formation of 1 umole of product (or the disappearance of 1 umole of substrate) per minute at 37°C except in the case of LDH and SDH where a unit was defined as 1 OD unit change per minute.

Specific activities are reported as units of enzyme per mg of protein. Enzyme enrichments are reported as specific activity of an enzyme in that fraction divided by the specific activity of that enzyme in the homogenate. Fractions to be assayed were sonicated to disrupt organized structures, (Ultrasonic Cell Sonicator, micro tip, max. power) on ice for 10 seconds. Preliminary experiments showed that recoveries were closer to 100% if the sample was adequately cooled.

CHEMICAL ASSAYS

Phosphate released was measured by a modification of the method of Fiske-Subbarow by Gomori (1942) which involves the use of methyl-ρ-aminophenol sulfate as the reducing agent. Protein was measured by a modification of the Lowry procedure (Lowry *et al*., 1951). The sample (0.2 ml vol.) was solubilized in 1 ml of 10% (w/v) SDS before following the usual procedure. Preliminary experiments showed that there was no effect on the chromophore except for dilution. Since the sample was routinely made up in 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM K_2HPO_4 (substances known to interfere with Lowry's method) the reagent blank routinely consisted of the sample suspending medium. Bovine serum albumin was used as a standard.

ELECTRON MICROSCOPY

Stock Solutions

Glutaraldehyde fixative-------2% glutaraldehyde
1 mM $MgCl_2$
50 mM K_2HPO_4, pH 7.0 with NaOH

Osmium post-fix---------------1% OsO_4
1 mM $MgCl_2$
100 mM K_2HPO_4, pH 7.0 with NaOH

Buffer------------------------1 mM $MgCl_2$
100 mM K_2HPO_4, pH 7.0 with NaOH

Poly-lysine-------------------0.05% poly-l-lysine bromide

Graded ethanol----------------30%
-50%
-70% (v/v) ethanol
-95%
-100%

Uranyl acetate----------------1% uranyl acetate in 95% ethanol

Lead citrate------------------as per Reynolds (1963)

Preparation of Samples for Scanning Electron Microscopy

8 mm diameter circular glass cover slips were cleaned by immersion in chromic-sulfuric acid cleaning solution, rinsed thoroughly with distilled deionized water and air-dried. Two drops of poly-lysine solution were placed on each slip and after 15 minutes at room temperature the slips

were rinsed with water. One drop of sample was placed on the spot made by the poly-lysine and incubated for 15 min. at $4^{o}C$. The slips were then put through th following fixation schedule:

	SOLUTION	TIME (mins.)	TEMP (^{o}C)
(1)	glutaraldehyde	30	4
(2)	buffer	15	4
(3)	buffer	15	4
(4)	osmium post-fix	30	4
(5)	buffer	15	4
(6)	buffer	15	4
(7)	30% ethanol	10	4
(8)	50% "	10	4
(9)	70% "	10	4
(10)	95% "	10	4
(11)	100% "	10	4
(12)	100% "	10	RT

The fixed, dehydrated slips were then glued to graphite stubs with silver paint and stored in a desiccator. The mounted slips were coated with 200Å of gold-palladium before observation in a Phillips 500 scanning electron microscope at 25 KV, 320Å spot size and 20° stage tilt.

GEL ELECTROPHORESIS

Electrophoresis of brain peptides was carried out in a 28 cm Slab Gel Apparatus (Hoeffer Scientific Instruments, San Francisco, Ca.) using a 0.75 mm thick gel and a 10 well sample comb. The conditions were those of Laemmli (1970), modified and briefly described here.

Component	separating gel	stacking gel	electrode buffer
Final pH	8.8	6.8	8.3
% acrylamide	10.0	3.0	---
% BIS	0.267	0.080	---
% TEMED	0.05	0.10	---
% K-persulfate	0.05	0.10	---
M Tris-base	0.375	0.125	0.025
% SDS	0.1	0.1	0.1
M Glycine	---	---	0.192

pH was adjusted with conc. HCl

The samples containing 1 mg of protein were made up to 1 ml in the following sample buffer:

62.5 mM tris, pH 6.8 (adjusted with HCl)
10% glycerol
2% SDS
5% BME
0.1% PMSF
0.0025% Bromphenol Blue

and incubated in a boiling water bath for 4 - 6 minutes, cooled, and 20 ul (containing 20 ug protein) was applied to each gel well. Standards were treated in the same manner. The gel was run at 10W constant power (starting conditions 270V @ 38ma) until the tracking dye reached the bottom of the gel (approximately 8 hours), the gel was removed and stained by the method of Fairbanks et al. (1971).

(1)	25% isopropanol 10% acetic acid 0.1% Coomassie brilliant blue	(1 hour)
(2)	25% isopropanol 10% acetic acid 0.001% Coomassie brilliant blue	(2x, 1 hour each)
(3)	10% acetic acid	(changed every hour until background is clear)

The timing of these washes is not strict and could be prolonged as long as overnight, however if kept in 10% acetic acid too long the low molecular weight components were leached from the gel. Gels could be permanently perserved by drying in a Bio-Rad Slab Gel Dryer (Bio-Rad Co., Metuchen, N.J.). The dried gels were photographed by transmitted light using Polaroid type 55 P/N film. Molecular weight standards chosen were: (Weber and Osborn 1969): phosphorylase A (100K),

bovine serum albumin (68K), glutamate dehydrogenase (53K), alcohol dehydrogenase (41K), chymotrypsinogen A (25.7K) and lysozyme (14.3K). Migration distances versus log[MW] were plotted for use as a standard curve.

RESULTS

Distribution of marker enzymes in primary fractions: In the primary fractions obtained by differential centrifugation of the homogenate containing 5 mM $MgCl_2$, the pattern of distribution of several marker enzymes is shown in Table 1. These results appeared anomolous when compared to previous work (Whittaker, 1959; Johnson and Whittaker, 1963). MAO, a mitochondrial marker appeared predominately in the P_1 (nuclei, cell debris) fraction, as did acid phosphotase, a lysosomal marker which would be expected to appear in the P_3 fraction. It also appeared that there was no separation of the activities, most of the activities and protein appeared in the P_1 fraction. This indicated unduly large sedimentation coefficients and aggregation of particles resulting in poor resolution. As mentioned earlier, high concentrations of divalent ions (Whittaker et al., 1964; De Robertis et al., 1963) and high ionic strength (Cotman, 1974) causes coacervation of particles. On the basis of this evidence, it was decided to lower the Mg^{++} concentration to 1 mM as used by several workers (Levitan et al., 1972). This change resulted in an enzyme distribution that was more in agreement with previous work and was retained as standard protocol.

Table 1 Distribution of Marker Enzymes among Primary Fractions obtained from Bovine Brain Homogenate. Numbers in parenthesis indicate the number of trials. Results are given as percent of total recovered activity in a fraction ± S.D. and the percentage of activity recovered from the homogenate ± S.D.

Fraction	P_1	P_2	P_3	S_3	Recovery
Protein (4)	64.6±6.6	13.4±3.4	4.4±2.0	17.5±3.0	103.5±18.5
Acid P (3)	58.6±4.5	12.3±1.3	3.0±2.4	26.1±1.3	100.1±10.1
Alk P (5)	77.5±3.7	9.3±2.6	5.4±2.5	7.8±3.2	99.9±24.1
MAO (5)	57.1±7.1	24.5±8.4	11.1±4.6	7.3±7.5	99.1±34.7
ACHE (5)	70.9±6.5	15.7±2.4	6.4±3.1	6.9±5.0	94.8±7.8
5'-nuc (4)	72.4±8.2	16.2±4.2	4.0±4.0	7.3±5.2	98.5±14.8

Table 2 Distribution of marker enzyme in P_2 fraction and P_2B and P_2C Fraction obtained by density gradient separation of P_2. Results are reported for P_2 as percentage of activity recovered from homogenate, and P_2B and P_2C as percentage recovered from P_2.

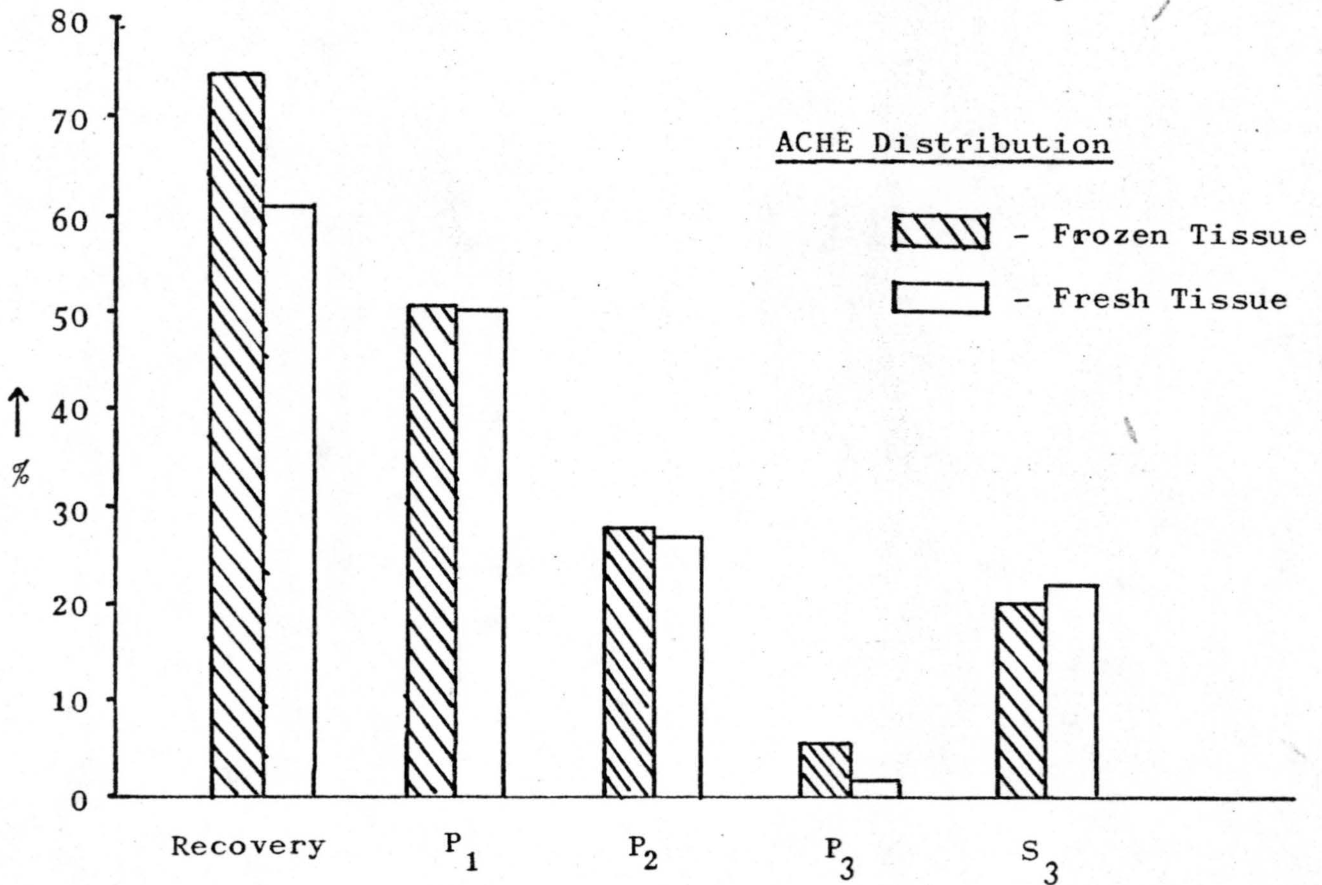
Fraction	Protein	Acid P	Alk P	ACHE	LDH	5'-nuc	MAO	SDH	NADH-cytc
P_2	66.7	17.2	3.3	22.3	12.5	50.0	38.2	26.1	44.5
P_2B	13.0	25.3	25.8	10.8	27.8	125.0	7.2	24.8	25.7
P_2C	4.5	12.3	16.3	6.6	16.7	62.5	15.4	26.7	24.6

Whole Brain Freezing Experiments: The distributions of ACHE and protein among the primary fractions for fresh and frozen brain are shown in Fig. 5. It is apparent that the differences between fresh and frozen brain are not great. It is also interesting to note that the recovered activity is higher in frozen tissue than fresh, probably due to the disruption of the tissue. At this time, fractions for enzyme assay were not disrupted by sonication.

Distribution ofEnzyme Markers in P_2, P_2B, and P_2C Fractions: Table 2 shows the pattern of enzyme markers in the P_2 fraction obtained from differential centrifugation of the homogenate and P_2B and P_2C fractions obtained from density gradient centrifugation of the P_2 fraction. The medium used here contained 1mM Mg^{++}. It is apparent that the results obtained using 1 mM Mg^{++} are more similar to previous work with a sizable quantity of MAO and SDH occuring in the P_2 fraction. Also approximately 12.5% of the LDH occured here indicating the presence of synaptosomes containing entrapped cytoplasm (Johnson and Whittaker, 1963). A substantial amount of Acid P. and NADH-cyt c appears here indicating the presence of lysosomal and microsomal contamination. Varing amounts of Alk P., ACHE, and 5'-nuc, putative membrane markers, were also found here. The density gradient

Fig. 5 Distribution of ACHE Activity and Protein among Primary Fractions. Results are reported as percentage of total recovered activity in each fraction and recovery from homogenate.

Protein Distribution



appeared to enrich the fraction in synaptoplasm in both cases while mitochondrial contamination was lessened according to both MAO and SDH. These fractions were also enriched in Acid P. and NADH-cyt c.

Scanning Electron Microscopy of Synaptosomes: In order to gain more evidence for the presence of intact synaptosomes, SEM was used to observe erythrocytes (control) and P_2C fraction. Human erythrocytes, kindly donated by the author, used as a control of the fixation procedure, and P_2C were both treated and observed as in the Methods section. The erythrocytes showed good morphology without shrinking or swelling. The P_2C fraction showed aggregated tissue with numerous particles, vesicular in nature, and of the size reported for synaptosomes (approximately 5000Å diameter; Morgan *et al.*, 1971). Spherical, highly electron reflective particles appearing to be 2000-4000Å in diameter, of unknown origin, were also observed in this fraction. See plates 1 through 4.

Failure of Ficoll-Sucrose Gradient: In collecting fractions from density gradients it is sometimes desirable to puncture the bottom of the tube and either allow the contents to drip out or displace the contents by pumping in a dense chase

solution and remove the contents from the top of the tube. If there is a pellet adhering to the bottom of the tube it will not loosen evenly and either contaminate the other fractions and/or not be recovered completely. This necessitated the use of a cushion which would be dense enough to keep the previously pelleted material from pelleting. A 2.2 M sucrose cushion proved useful but had the following drawbacks: (1) 2.2 M sucrose is highly hypertonic to brain particles and would be subject to the limitations discussed earlier which caused the switch to ficoll in the first place, and (2) the sucrose diffused into the lowest ficoll-sucrose band causing particle bands to be less resolved. A 25% (w/v) ficoll in SMP cushion was tried with the resulting tube shown diagramatically in Fig.6. Apparently some mixing occured between the 16 and 25% ficoll solutions resulting in no separation of P_2C and P_2D fractions. It was thought that this occurred due to mixing during acceleration and/or deceleration of the tube, of a self-forming gradient characteristic of ficoll similar to CsCl. These hypotheses were tested by layering gradients and fractionating them at various times in the procedure. The results can be seen in Fig. 7. It can be seen that gradient recovery

Fig. 6 Diagram of Ficoll-Sucrose Density Gradients.

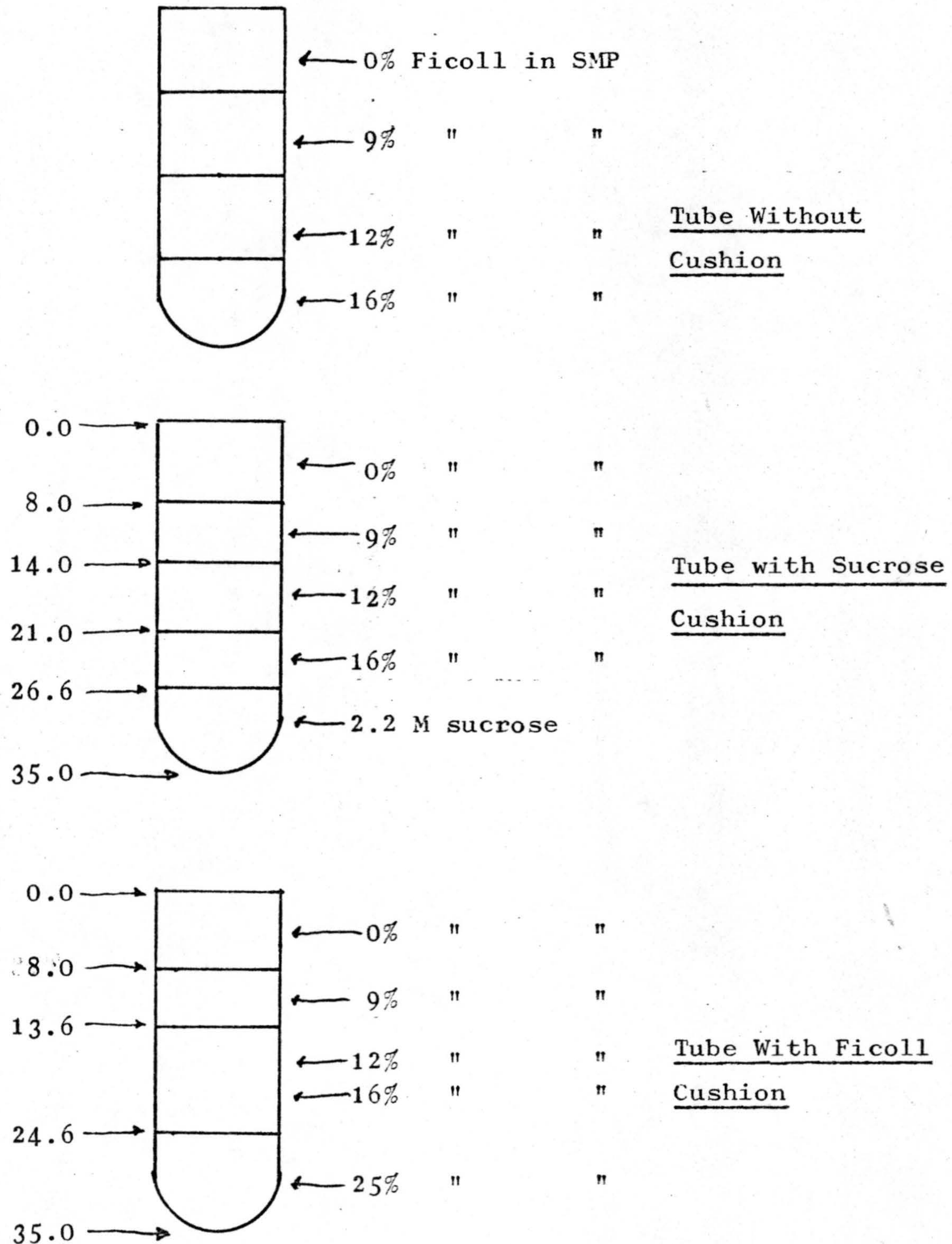


Fig. 7 Recovery of Ficoll-Sucrose Gradients.

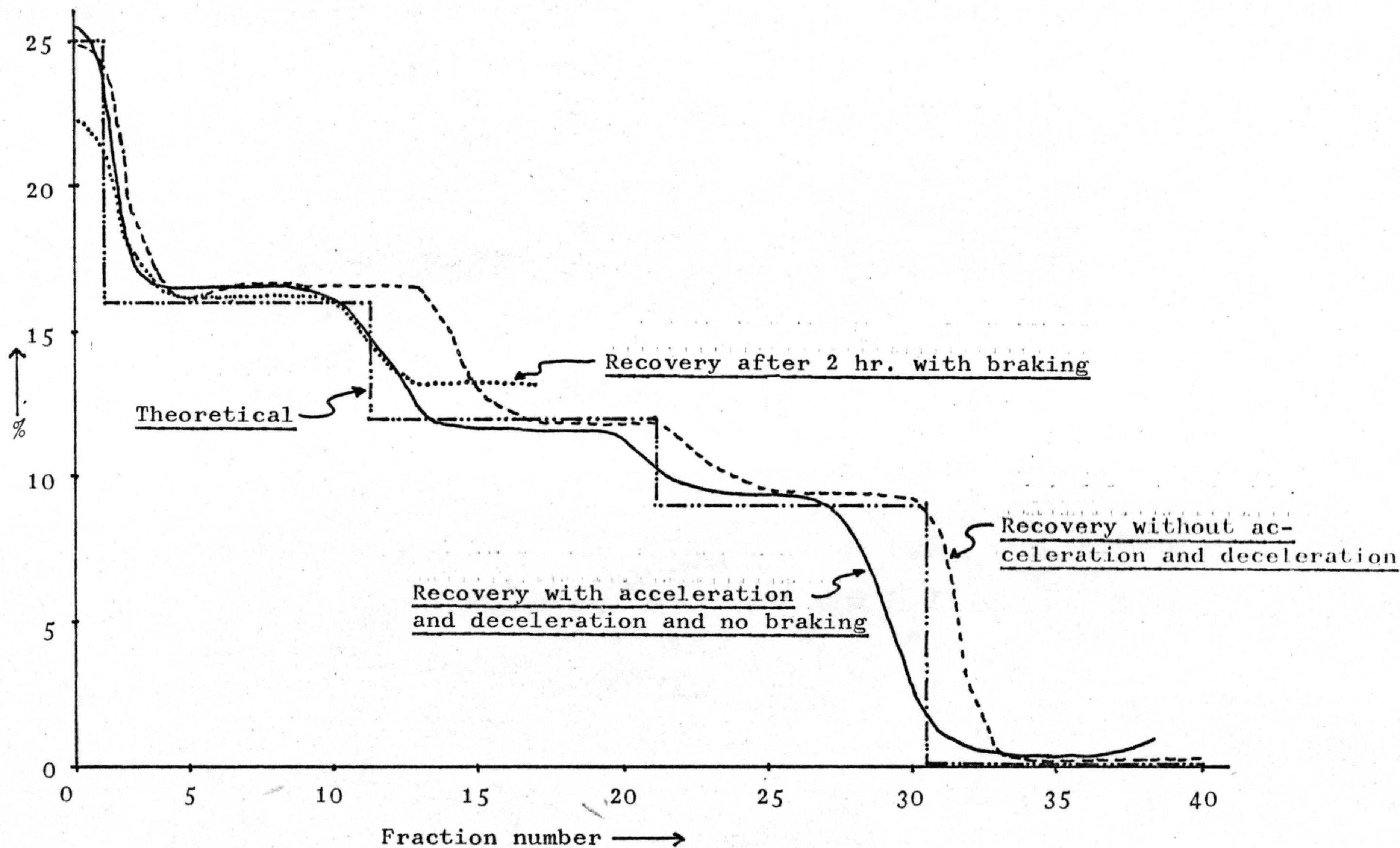
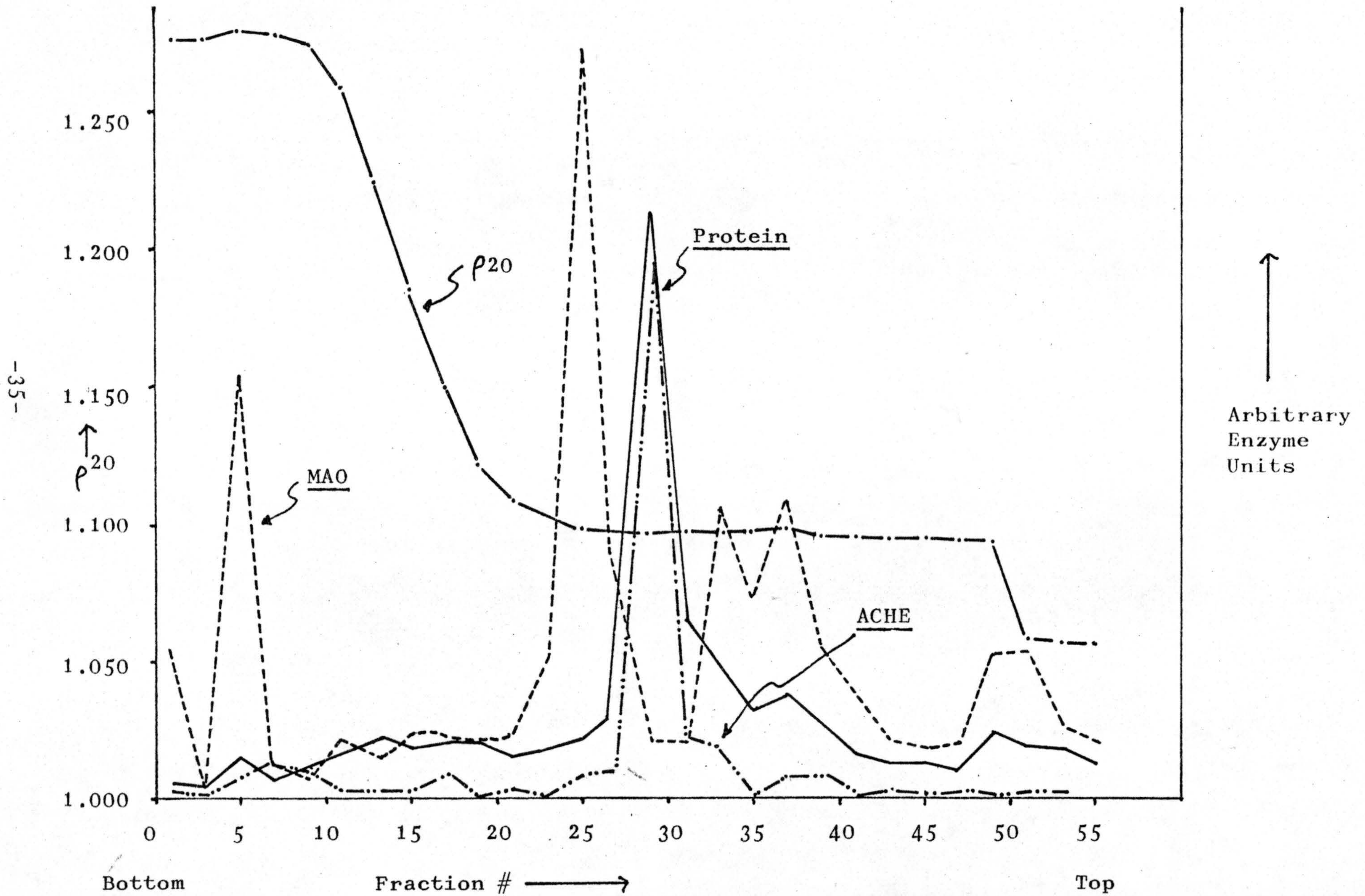


Fig. 8 Output from SZ-14 Rotor.



is the same (except for slight diffusion effects and mixing due to turbulence) whether the gradient was fractionated (1) immediatly after forming; (2) after acceleration to 21,000 rpm (55,000 G) and decelerating with or without braking; or (3) after two hours at 21,000 rpm.

SZ-14 Reorienting Gradient Zonal Rotor: Fig. 8 shows the results of the fractionation of a P_2 fraction using the Sorvall SZ-14 rotor. ACHE is effectivly separated from MAO yielding comparable results to the separation performed in the SW 27 rotor except scaling-up approximately elevenfold.

Disruption of Synaptosomal fractions by Osmotic Shock: Table 3 shows the distribution of marker enzymes following osmotic shock of the combined P_2B and P_2C fractions and subsequent centrifugation. Small amounts of Acid P, Alk P and SDH were released by this treatment, while substantial amounts of NADH-cyt c, 5'-nuc and ACHE were released. Only 30% of the LDH was released as contrasted with 75% reported by Johnson and Whittaker (1963). This could be due to incomplete rupture of the particles or LDH adhering to membranes.

Table 3 Distribution and Recovery of marker enzymes following osmotic shock of the synaptosomal fraction. Experimental details as in text. Results are reported as in Table 2.

Fraction	Protein	Acid P.	Alk. P.	ACHE	LDH	5'-nuc	MAO	SDH	NADH-cyt c
W_s	9.6	6.4	9.5	41.4	29.8	54.7	30.8	5.9	49.2
W_p	90.4	93.6	90.5	58.6	70.2	45.3	69.2	94.1	50.8
Recovery	89.6	97.1	291.3	100.0	90.5	99.9	150.7	63.0	87.8

Table 4 Distribution and recovery of marker enzymes in fractions prepared from disrupted synaptosome fractions by density gradient separation. Results expressed as in Table 2.

Fraction	Protein	Acid P.	Alk. P.	ACHE	LDH	5'-nuc	MAO	SDH	NADH-cyt c
W_pA	1.4	UD	UD	UD	5.7	4.3	3.9	UD	UD
W_pB	36.0	63.1	50.3	62.5	32.1	95.7	19.1	UD	85.4
W_pC	27.4	18.7	11.0	21.9	34.9	UD	30.7	UD	14.6
W_pD	10.8	5.1	7.9	UD	21.7	UD	13.6	UD	UD
W_pE	24.5	12.5	30.9	15.6	5.7	UD	32.7	100.0	UD
Recovery	74.0	86.6	37.0	188.2	83.5	138.6	300.9	83.5	89.2

Fractionation of Disrupted Synaptosome Fractions on Density Gradients: When the disrupted synaptosomal fractions were separated by discontinuous sucrose gradient centrifugation, the pattern shown in Table 4 was obtained. The low recoveries were due to mechanical manipulation; however, the recoveries of ACHE, 5'-nuc and MAO are harder to explain.

Electrophoresis of Fractions: The fractions from the discontinuous ficoll-sucrose and discontinouos sucrose gradients were subjected to the discontinouos SDS-polyacrylamide electrophoresis technique of Laemmli (1970). The results are shown in Plate 5 and Table 5. Proteins were included as molecular weight standards by which molecular weights of the proteins in the brain fractions might be determined. Fig. 9 shows the migration distance of each protein versus the logarithm of the molecular weight which should yield a straight line. Eighteen bands were selected from the brain fraction, the molecular weights were calculated from the standards, and relative staining intensities were estimated visually. It is apparent that while few of the proteins are unique to any single fraction (indicating incomplete resolution by the density gradients) there are definite trends of intensities lending evidence towards the

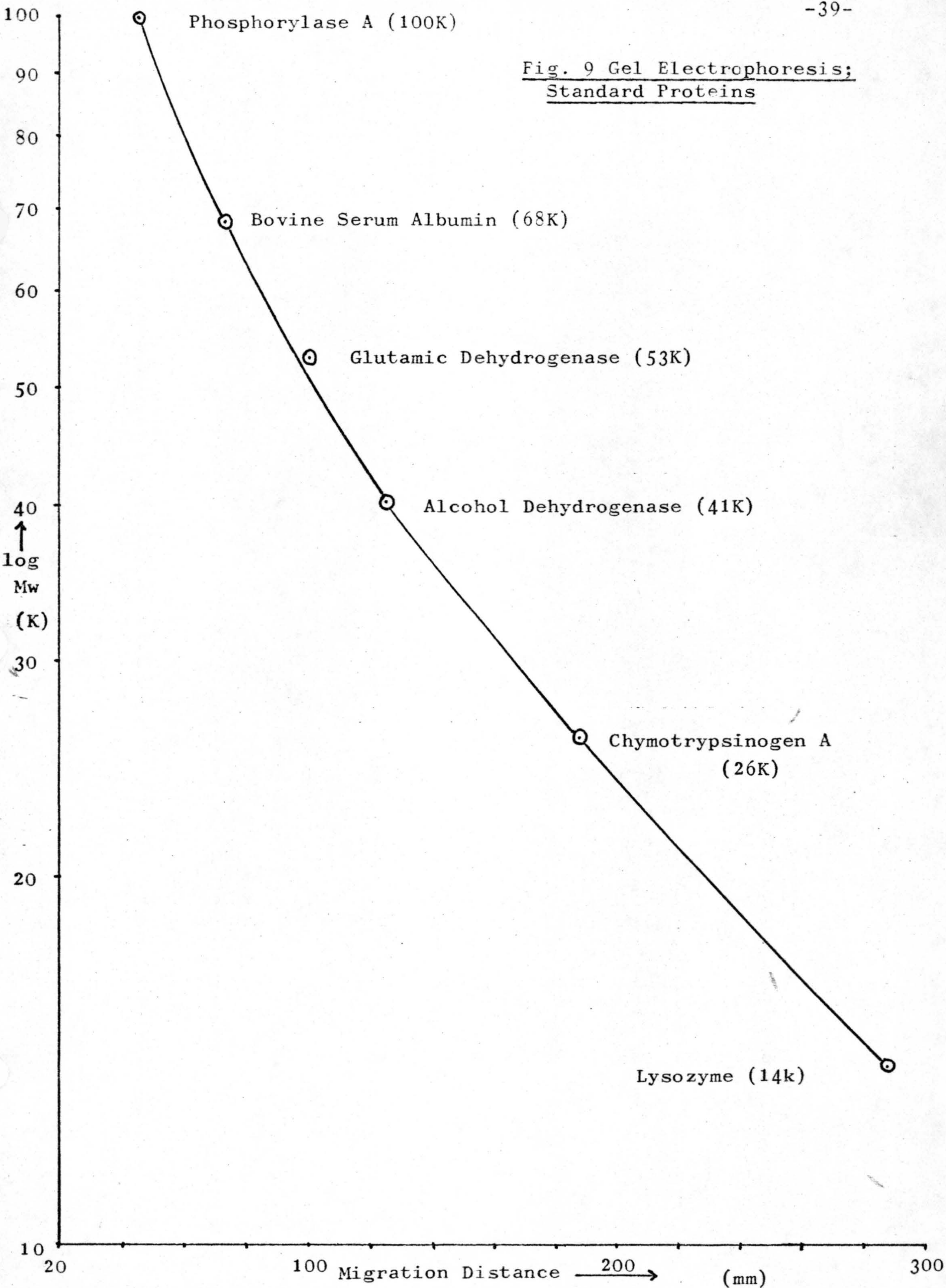


Fig. 9 Gel Electrophoresis; Standard Proteins

Table 5 Selected peptides derived from brain fractions. Staining intensities: +, ++, +++, ++++, increasing intensity; - undetected.

Component #	Migration distance (mm)	Calculated MW	P_2A	P_2B	P_2C	P_2D	W_pA	W_pB	W_pC	W_pD	W_pE
1	43	87	+	++	+++	++++	-	+	+	++	++++
2	67	74	+	++	+++	++++	-	+	+	++	+++
3	73	68	+	++	+++	++++	-	+	+	++	+++
4	80	63	-	+	++	+++	-	-	+	++	+++
5	100	53	+	++	+++	++++	+	++	++	++	++++
6	107	49	++	++	++	++++	+	+	+	++	++++
7	112	46	++	++	++	++++	+	++++	+++	++	+
8	118	44	+	++	+++	++++	-	+	++	+++	++++
9	153	33	+	+	++	+++	+	++	++	++	+++
10	168	30	+	++	+++	++++	-	-	+	++	+++
11	185	26.5	-	+	++	+++	-	-	+	+	++++
12	205	23.0	+++	++	+	+	+	++++	++	+	-
13	235	18.5	+++	++	+	+	+	++++	++	+	-
14	255	17.0	++++	+++	++	++	++	++++	+++	++	+
15	258	16.7	++	++	++	++	+	++++	++	+	+
16	274	15.3	-	-	-	+	-	-	-	-	+
17	284	14.3	-	-	-	-	-	-	-	-	+
18	298	13.1	-	-	-	-	-	-	-	-	+

localization of certain proteins in specific fractions. These estimations, while not absolute, are useful since approximately the same amount of protein was placed in each well and the banding intensities are, in some cases, very different.

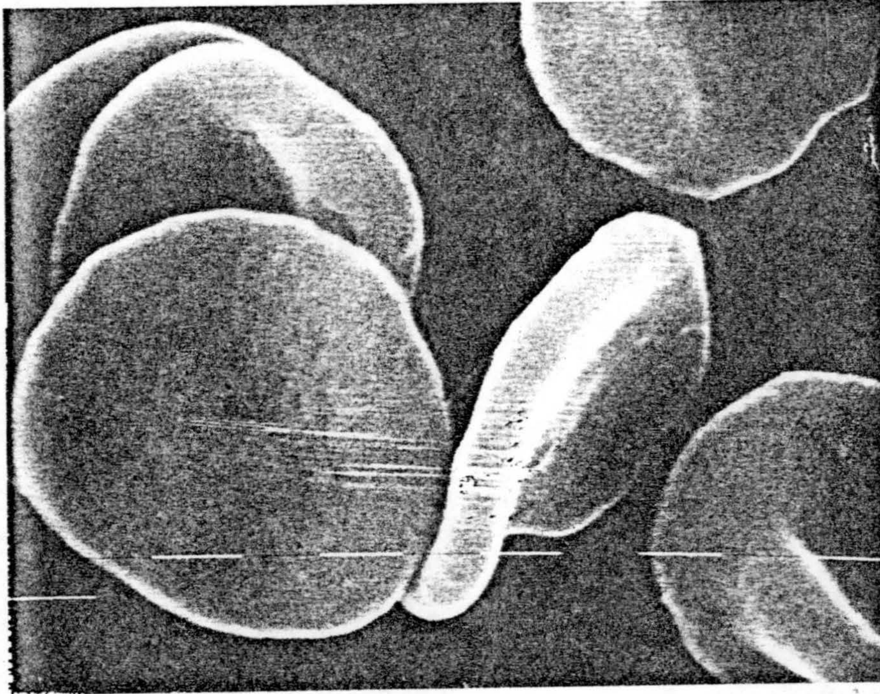


Plate 1
Human Erythrocytes
10,000 X, 640Å
spot size, 650Å
gold-palladium
coating.

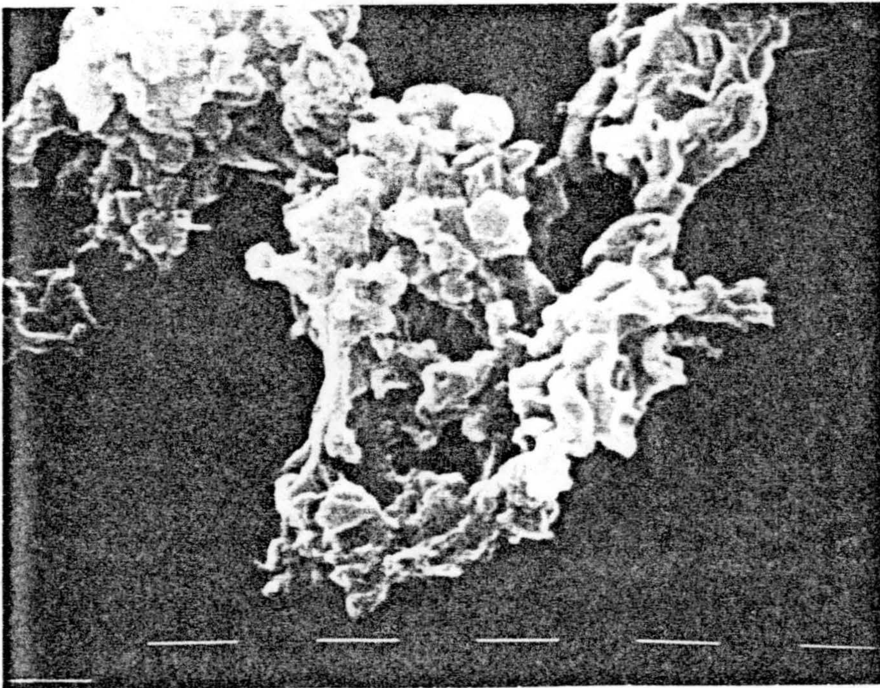


Plate 2
Synaptosomes,
10,000 X,
P_2C fraction.

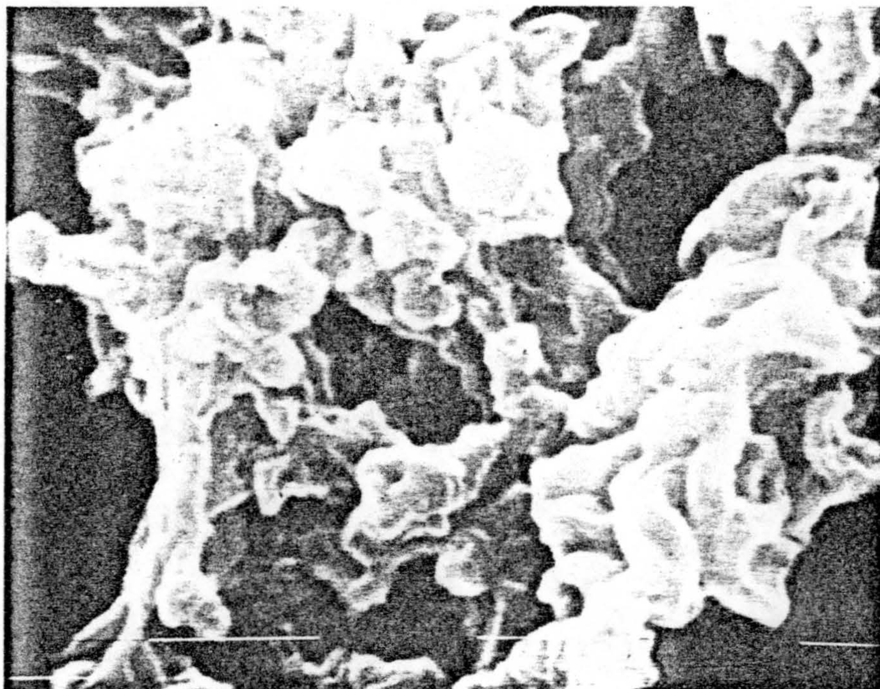


Plate 3
Synaptosomes, 20,000 X

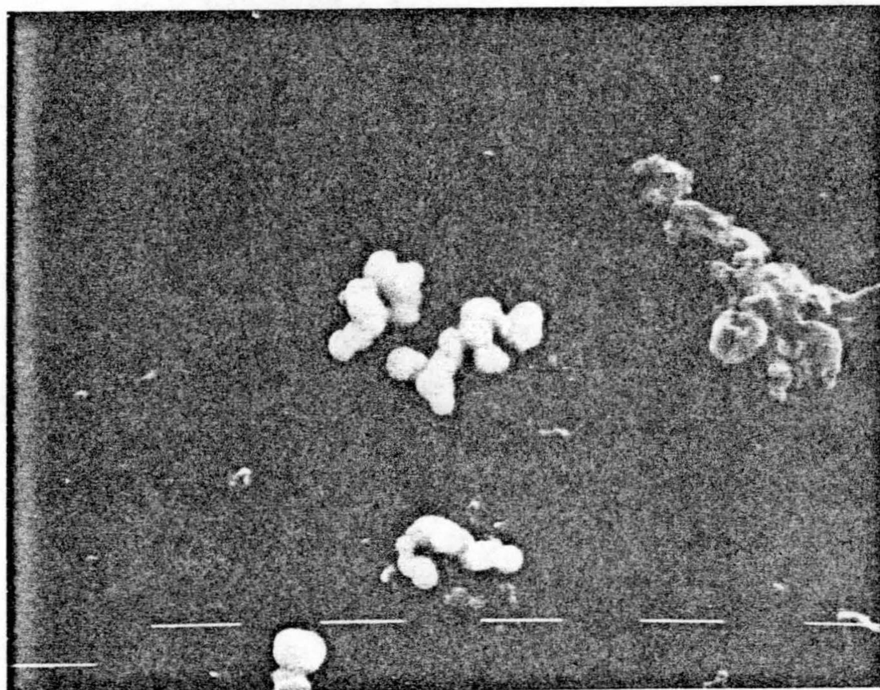


Plate 4
Unidentified particles in P_2C, 10,000 X

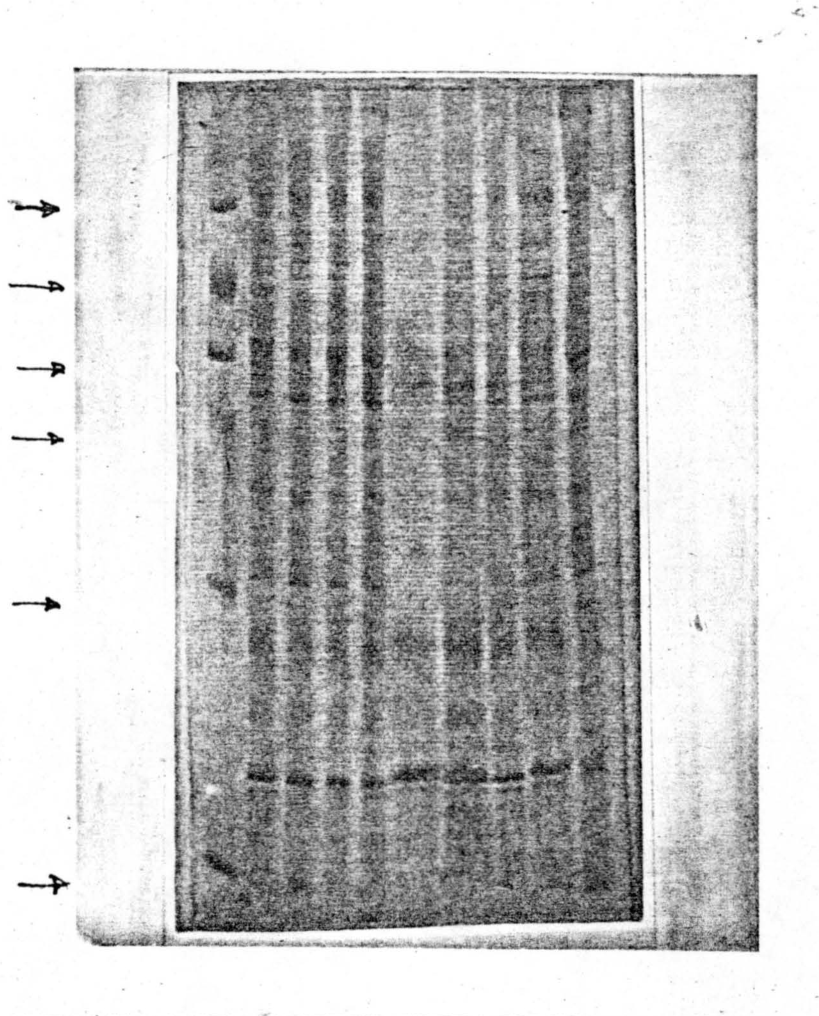


Plate 4 Gel electrophoresis of brain peptides, migration is from top to bottom. Samples listed from left to right: standard proteins, $P_2A, P_2B, P_2C, P_2D, W_pA, W_pB, W_pC, W_pD, W_pE$. 20 ug of protein were applied to each sample well.

DISCUSSION

Bovine grey matter was chosen for this work because of the large quantities which are readily available and the higher density of synaptic endings known to exist in this portion of the brain relative to the whole brain. Therefore, large amounts of brain may be processed with a good yield without having to compromise the purity of the resultant fractions by using whole brain.

While the distributions of protein and ACHE activity showed little change in the primary fractions derived from fresh and frozen brain, more evidence supporting the use of this preservative step is necessary. Obviously, electron microscopy of intact tissue and the resulting synaptosomes will yield information but LDH distribution can serve to indicate the percentage of synaptosomes obtained. Whittaker (1959) has shown that freezing and thawing releases acetylcholine from crude mitochondrial fractions derived from guinea pig brain. This could discourage the hope of freezing brain except that many cycles of freeze/thaw are required to disrupt the fractions (30 cycles release 60% of bound ACH) and that on observation with transmission EM it was found that synaptic vesicles were preferentially disrupted

by this treatment, not synaptosomal membranes. It would seem, therefore, that synaptic membrane isolation would not be seriously perturbed by one cycle of freeze/thaw.

In an attempt to find an optimum homogenization medium for bovine brain it has been determined that 1 mM $MgCl_2$ affords an acceptable (although not necessarily optimal) medium additive. As mentioned earlier, buffer concentrations greater than 10 mM almost invariably result in aggregation of particulates, and it has been determined in this paper that 5 mM Mg^{++} gives similar results. It is interesting that while some workers advise (even insist) on the presence of some small concentration of divalent metal ion (Levitan _et al._, 1971), all maintain that their medium preserves morphology the best! The medium utilized here (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM K_2PO_4, pH 7.6 by HCl) was decided on the basis of the preliminary results obtained and _a priori_ reasoning. The pH was decided on as it is the intracellular pH and will tend to preserve the cellular environment, buffered with a minimum of physiologically compatible salt. It remains to be seen whether this system is optimal.

The differential centrifugation procedure was adapted from that of Morgan _et al._ (1971) and Rodriguez DeLores Arnaiz

et al.(1967) with modification to attain a P_2 fraction in a reasonable time. The large contamination of P_2 by lysosomal and microsomal membrane fragments could certainly be reduced by more washes of the P_2 pellet, but this would result in a longer preparation time and loss of some synaptosomes due to disruption incurred by the pelleting and resuspension operations. It is possible that larger synaptosomes are sedimenting in the P_1 fraction unnoticed since this fraction was not assayed. Certainly, systematic studies are necessary to determine optimum cut-off for the centrifugal fields creating these divisions.

The Ficoll-sucrose density gradient was useful in removing contamination by mitochondria but less so with the lysosomes and endoplasmic reticulum membrane which band at approximately the same density as synaptosomes (see Figure 4). As mentioned earlier, reduction of small membrane fragments in the primary fractionation steps would be useful. Mitochondrial contamination could probably be reduced even further by weighting the mitochondria with SDH produced formazan as in the method of Cotman and Taylor (1972). The 5'-nucleotidase activity shows an unusual enrichment in the P_2B fraction with an inconceivable recovery of 125% of the P_2 activity. The

only possible explanation for this that I can put forth is that when samples are taken for enzyme assay from incompletely homogenized fractions a pipette may pick up an unusually large fragment of tissue thereby not giving a representative estimate of that assay.

The scanning electron micrograph showed the presence of vesicular particles but this was obscured by the large amount of aggregated material. This was most likely due to the high concentration of phosphate in the gluteraldehyde fixative. The phosphate was included both to buffer the fixative and to bring the osmolarity to approximately 320 mOSM. It would prove useful to lower the concentration of this component and perhaps remove it completely, replacing the phosphate with 200 mM sucrose and adjusting the pH to 7.0 with dilute NaOH.

On plate 4 unusual particles are shown which were seen in the P_2C fraction. The origin of these is unknown. Although they are in the size range of synaptosomes, it is unlikely that they would have escaped the aggregation which the vast majority experienced. They are too large to be synaptic vesicles but may be lysosomes or zyomogen granules. It remains to be seen, however, whether these granules are

repeatedly found in these fractions.

The reason for the failure of the Ficoll-sucrose cushion is still unknown. Turbulence during the centrifugation run or a possible self-forming gradient effect have been ruled out. Or, as Garey _et al_. (1972) mentioned, it could be an "overloading" of the gradient with the resultant mixing at the interface due to the discontinuity. However, there is no apparent reason why the 16-25% interface should be more sensitive than the others.

The SZ-14 rotor appears to be a useful instrument for membrane preparation. In order to get an appreciable yield of SPM's, large amounts of brain must be processed. The GSA superspeed rotor is useful for the preliminary fractionation steps but a SW 27 tube will hold, at most, the P_2 fraction derived from 4 grams of brain tissue. Of course, several tubes can be prepared but this can take an appreciable amount of time and the fractionation of each tube is subject to loss. It would seem better if a large amount of P_2 fraction could be handled in one batch. This paper describes the processing of 100 ml of P_2 fraction from greater than 30 gm of grey matter in a single batch. Theoretically, 300 ml of P_2 could be separated.

Osmotic shock of the synaptosomal fractions P_2B and P_2C yielded several interesting results. Little (less than 10%) of the Acid Phos, Alk Phos and SDH activity went into the shock supernatant (W_s) while roughly half of the NADH-Cytochrome C oxidoreductase, 5'-nuc and ACHE activity was released upon the treatment. Slightly lesser amounts (less than 30%) of MAO and LDH activities were released. The release of ACHE and NADH-Cytochrome C would seem to represent disruption of microsomes resulting in a decrease in contamination by the organelles. Also, it is apparent that lysosomes and the internal membrane of mitochondria are stable to this treatment while an appreciable amount of the outer mitochondrial membrane is detached. Since only 30% of the LDH activity is released, either the majority of the synaptosomes remain undisrupted or the LDH is adhering to some organelles. Increasing the ionic strength of the shocking medium should differentiate between these two possibilities unless the synaptosomal membrane proves sensitive to the change, which is unlikely since synaptosomes are routinely suspended in Kreb's buffer for metabolic studies (Bradford, 1969) and in preparation for negative phosphotungstinate staining. MAD and

Alk. Phos. show the same anomolous recoveries as mentioned earlier for 5'-nuc. It may prove useful for purer fractions to fractionate the W fraction three ways: W_{p1}, material from fraction W sedimenting at 11,500 G x 25 min (i.e., that material whose sedimentation properties remain unchanged after osmotic shock), W_{p2} pelleted by 40,000 to 100,000 G x 60 min (i.e., membrane fragments, released by shock), and W_s unsedimented by the previous G forces (i.e., soluble protein released by shock). Morgan et al. (1971) follow a procedure similar to this but do not show the pattern of activities obtained.

Separating the osmotic shock pellet on a sucrose gradient yields the final synaptic plasma membrane fraction, W_p. This fraction had the highest recovered activities of Alk Phos, ACHE and 5'-nuc, several putative membrane markers. It also had the highest recovery of Acid Phos. and NADH-Cytc. There was no detectable SDH activity but considerable MAO indicating the presence of outer mitochondrial membranes. Again there is this problem of anomolous recoveries of ACHE, 5'-nuc, and MAO.

It is possible to correct somewhat for the anomolous behavior of these membrane markers by taking advantage of

the redundancy of the enzyme data. Take, for example, the case of alkaline phosphotase after osmotic shock (see Table 3). There is apparently a three-fold activation of the activity after shock which seems unreasonable since all of the fractions to be assayed are sonicated to prevent such an occurrance. The problem is probably, as mentioned before, due to large tissue fragments being caught in the transfer pipette used for the assay. It would appear that the W_p assay was affected like this, and we can compensate by assuming a 100% recovery (0.080 units of enzyme were shocked, 0.023 units resulted in the W_s fraction, then we can calculate 0.057 units resulting in W_p contrasted with 0.21 units assayed in W_p). It would then appear that the distribution would be W_s: 28.7% and W_p: 71.3% of the recovered activity (100% recovery). This is in agreement with the recovery of Alk. Phos. from the subsequent density gradient (i.e. 37.9%). Assuming that 0.057 units were applied contrasting with the 0.21 units thought to have been applied, the recovery now is calculated as 136.1%. This figure is apparently too high probably resulting from the same problem but it is impossible to point to any specific fraction as being poorly assayed.

In the case of 5'-nucleotidase the recovery from the density gradient of 138% can be corrected to the appropriate 80% recovery (the recovery is chosen as 80% and not 100% to agree with the protein assay which is expected to reflect the true recovery of the system and not to be affected by any activation or deactivation). The W_p fraction applied to the gradient contained 0.75 units of activity, the W_{pA} contained 0.050, the W_{pB} contained 1.00, and in the rest of the fraction, activity was not detected. W_{pB} can be corrected to 0.55 units resulting in the following: 100.0% recovery, W_{pA} = 8.4%, W_{pB} = 91.6% of the recovered activity. However, it is not possible to correct for the 125% recovery of the activity in the P_2B fraction from the P_2 fraction since not all of the fractions were assayed and recoveries couldn't be calculated.

Finally the acetylcholinesterase activity could also be corrected in this manner. The total recovery from the final density gradient could be brought to 80% of the activity of the W_p fraction by adjusting the W_{pB} fraction to 0.004 units (yielding 0.030 units on the gradient which is 80% of the 0.037 units found in the W_p fraction).

Given this method of correction, the final membrane was

enriched over the homogenate 12.4 times in 5'-nucleotidase, 1.2 times in alkaline phosphotase, and 0.096 times in acetylcholinesterase. The 5'-nuc. enrichment looks more reasonable but I seriously doubt that the ACHE would be that low even though it is not specifically located in SPM.

The electrophoresis data are interesting on two points. Firstly, peptides were found migrating at approximately the same rate as brain actin (Blitz & Fine, 1974) at 45K. While the estimated Mw's don't correspond exactly, I would estimate a $\pm 5\%$ spread in the determined Mw yielding peptides labeled #7&8 as within the range. Secondly, the staining intensities can be used to localize a given peptide in a fraction. Since the total amount of protein applied to each sample well was the same, a peptide would stain most heavily in the fraction where it predominates. For example, components 1 through 4 appear most concentrated in W_pE while # 7 and 12 through 15 predominate in W_pB.

The standards curve shows some non-linearity in the relationship between log Mw and migration distance. This may be due to the high concentration of persulfate and TEMED used to polymerize the gels.

As was mentioned earlier, intact synaptosomes which

are useful for metabolism and uptake studies are available as a relatively impure population. It would be desirable to purify these vesicles without disrupting them. It may be possible to accomplish this by membrane filtration utilizing Uni-Pore membrane filters (Bio-Rad). These are available in a variety of sizes ranging from 8u to 0.2u (pore sizes). A possible protocol would consist of a filtration of the P_2 fraction through a 1 or 0.8u membrane to remove material larger than synaptosomes, then passing the filtrate through a 0.4 or 0.2u membrane to retain synaptosomes (and unfortunately mitochondria). The 0.4u membrane would probably result in a purer fraction but a lower yield and it is possible that there may be a separation of cholinergic and non-cholinergic synaptosomes on the basis of size. Following the filtration steps, the Ficoll-sucrose gradient would follow as previously described yielding a (probably) purer synaptosome population.

SUMMARY

(1) Synaptosomes and sunaptic plasma membranes have been isolated from Bovine cerebral cortex, as shown by electron microscopy and occluded Lactate dehydrogenase.

(2) The major contaminants of the membranes appear to be membrane fragments derived from outer mitochondrial membranes and lysosomes, and to a lesser extent from microsomes.

(3) The final membrane preparation was enriched 12 times in 5'-nucleotidase and 1.2 times in alkaline phosphotase, putative membrane markers.

(4) Procedures are outlined for the improvement of the preparation.

(5) A procedure is suggested for the further purification of synaptosomes using membrane filters.

(6) Electrophoresis shows protein bands in the final membrane preparation migrating at approximately the same rate a muscle and brain actin.

APPENDIX Units of Activity

Fraction	Protein (mg)	Acid P.	Alk. P.	ACHE	LDH	5'-nuc	MAO	SDH	NADH-cyt c
Homogenate	900	8.80	5.80	7.49	360	8.0	80.2	2.01	1.24
P_2	600	1.46	0.19	1.67	45.0	4.0	30.6	0.525	0.552
P_2B	78.1	0.37	0.049	0.18	12.5	5.0	2.2	0.130	0.142
P_2C	26.9	0.18	0.031	0.11	7.5	2.5	4.7	0.140	0.136
W_s	9.05	0.034	0.023	UD	5.4	UD	3.2	0.010	0.120
W_p	85.0	0.50	0.21	0.17	12.7	3.4	7.2	0.16	0.124
W_pA	0.86	UD	UD	UD	0.60	0.20	0.84	UD	UD
W_pB	22.6	0.276	0.039	0.16	3.40	4.5	4.1	UD	0.094
W_pC	17.2	0.081	0.0085	0.070	3.70	UD	6.6	UD	0.016
W_pD	6.8	0.022	0.0061	UD	2.30	UD	2.9	UD	UD
W_pE	15.4	0.054	0.024	0.050	0.60	UD	7.1	0.13	UD

UD - activity undeteeted

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